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Diazoxide and dimethyl sulphoxide alleviate experimental cerebral hypoperfusion-induced white matter injury in the rat brain

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Abstract

Aging and dementia are accompanied by cerebral white matter (WM) injury, which is considered to be of ischemic origin. A causal link between cerebral ischemia and WM damage has been demonstrated in rats; however, few attempts appear to have been made to test potential drugs for the alleviation of ischemia-related WM injury.

We induced cerebral hypoperfusion via permanent, bilateral occlusion of the common carotid arteries of rats. A mitochondrial ATP-sensitive potassium channel opener diazoxide (5 mg/kg) or its solvent dimethyl sulphoxide (DMSO) was administered i.p. (0.25 ml) on 5 consecutive days after surgery. Sham-operated animals served as control for surgery, and non-treated rats as controls for treatments. Thirteen weeks after surgery, the animals were sacrificed and astrocytes and microglia were labeled immunocytochemically in the internal capsule, the corpus callosum and the optic tract.

The astrocytic proliferation was enhanced by cerebral hypoperfusion in the optic tract, and reduced by diazoxide in DMSO, but not by DMSO alone in the corpus callosum. After carotid artery occlusion, microglial activation was enhanced two-fold in the corpus callosum and four-fold in the optic tract. DMSO decreased microglial activation in the optic tract, while diazoxide in DMSO, but not DMSO alone, restored microglial activation to the control level in the corpus callosum.

In summary, the rat optic tract appeared to be particularly vulnerable to ischemia, while the effect of diazoxide was restricted to the corpus callosum. We conclude that diazoxide dissolved in DMSO can moderate ischemia-related neuroinflammation by suppressing glial reaction in selective cerebral WM areas.

Keywords: Astrocyte; Cerebral hypoperfusion; Diazoxide; Dimethyl sulphoxide; Microglia; White matter

Cerebral white matter lesions have been associated with the progression of aging and cognitive impairment [2,4]. Further, these lesions have been suggested to originate from a variety of vascular causes ranging from hypertension to cerebral microinfarcts and ischemia [5,7]. To support the ischemic theory of white matter injury, experimental animal models have been employed, such as bilateral occlusion of the common carotid arteries of rats [8,17,18]. The findings of such studies have compellingly demonstrated that chronic cerebral hypoperfusion can initiate a wide array of neuropathological white matter changes. For instance, axonal degeneration, myelin and oligodendrocyte damage, astrogliosis and microglial activation have been identified in the optic tract and the corpus callosum of rats with occluded carotid arteries [8,17,18]. Our own results emphasized the specific involvement of the optic tract in ischemic white matter damage in the rat brain, and pointed to the marked proliferation of the astrocytes and to the activation of the microglia in the region [8].

A number of pharmacological compounds have been considered to alleviate neuronal damage after ischemic insults in the brain, but not too much is known about the pos-

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sible means of limiting ischemia-related white matter injury. A couple of studies have demonstrated that nimesulide, a cyclooxygenase-2 inhibitor, and ibudilast, a phosphodiesterase inhibitor succesfully limited white matter injury in the ischemic rat brain shortly after carotid artery occlusion [19,20].

We have recently demonstrated the neuroprotective effect of diazoxide and its solvent dimethyl sulphoxide (DMSO) in chronic experimental cerebral hypoperfusion [9]. Diazoxide is a putative, mitochondrial ATP-sensitive potassium channel opener [1] that has proved to be neuroprotective in several ischemia models. For example, pretreatment with diazoxide reduced infarct size after middle cerebral artery occlusion in experimental animals [12,16], and preserved the neuronal viability in cell cultures after glucose–oxygen deprivation [11]. Our previous study has predominantly demonstrated that diazoxide cannot only act on neurons but also prevents ischemia-induced, long-term microglial activation [9]. On the other hand, its solvent DMSO is itself also known to possess vascular and neuroprotective properties [9,14].

In the present study, we set out to evaluate the potential protective effects of diazoxide and DMSO on the glial compartments of the cerebral white matter in a rat model of chronic cerebral hypoperfusion.

The experiments were approved by the ethical committee of the University of Szeged, Hungary. Fifty-one male Wistar rats (210 ± 10 g) were anesthetized with 400 mg/kg chloral hydrate i.p., followed by 0.05 ml atropine i.m. Experimental cerebral hypoperfusion was induced by permanent, bilateral occlusion of the common carotid arteries (2VO) in 26 animals [9]. The same surgical procedure was performed in the control group (SHAM), but without the actual ligation. The survival rate was 78.4%.

Both groups were divided into three subgroups ($n = 6$) on the basis of the postsurgical treatment, as described earlier [9]. The first set of animals was treated with 5 mg/kg diazoxide given in 0.25 ml DMSO, i.p. (SHAM-DMSO + DZ, 2VO-DMSO + DZ). The second set of animals of both SHAM and 2VO groups received 0.25 ml DMSO, i.p. (SHAM-DMSO, 2VO-DMSO, respectively). The last set of animals received no postoperative treatment and served as controls (SHAM-non-treated, 2VO-non-treated). The animals were injected on 5 consecutive days in the initial phase of cerebral hypoperfusion. The first injection was applied directly after surgery.

Thirteen weeks later, the animals were anesthetized with an overdose of pentobarbital, and perfused transcardially with 100 ml saline, followed by 400 ml 3.5% paraformaldehyde and 0.5% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed, and one hemisphere was post-fixed in the fixative solution for up to 1 h.

Immunocytochemical staining was performed as described earlier [8]. Briefly, free-floating coronal sections at Bregma −3.14 mm were cut at 20 μm thickness on a cryostat microtome. Slices were stained immunocytochemically for glial fibrillary acidic protein (GFAP) to visualize astrocytic proliferation. The samples were incubated in a primary antibody solution containing mouse anti-GFAP antibody (Sigma), 1:200, 1% normal sheep serum, and 0.3% Triton X-100 in 0.01 M PBS. The secondary antibody solution consisted of sheep anti-mouse biotinylated IgG (Jackson), 1:200, and 0.3% Triton X-100 in 0.01 M PBS. Finally, the sections were incubated in HRP-Streptavidine (Zymed), 1:200, and the color reaction was conventionally developed with diaminobenzidine (DAB) and H_2O_2.

Microglial activation was visualized over the white matter areas with the cell surface marker CD11b (OX-42). The sections were incubated in a primary antibody solution containing biotinylated mouse anti-CD11b antibody (OX-42, Serotec), 1:500, 20% normal swine serum (NSS), and 0.03% merthiolate in 0.01 M PBS. Next, the sections were incubated in a solution of STA-PER (Jackson), 1% NSS, and 0.03% merthiolate in 0.1 M Tris buffer. Finally, the color reaction was developed by nickel-DAB and H_2O_2.

The percentage surface area of GFAP-positive astrocytes was quantified in the medial corpus callosum and the internal capsule; in the optic tract, relative optical density was computed instead of the percentage area, since the homogenous labeling did not permit area measurements. As regards the OX-42 labeling, the percentage surface area was measured for all three regions of interest (Olympus BX50, DP50, software: ImagePro Plus, Media Cybernetics). As in our previous protocol [8], three consecutive coronal sections were selected for analysis. Regions of interest were delineated manually at 10× magnification. The area covered by GFAP-or OX-42-positive glia was computed as a percentage of the total area delineated. The measured results on the three sections per animal were averaged and the average values were used for further statistical analysis. The data were analyzed by a two-way ANOVA model of the software SPSS.

The internal capsule was unaffected by either cerebral hypoperfusion or the pharmacological treatment.

In the medial corpus callosum, astrocyte proliferation was not enhanced by cerebral hypoperfusion, but the postsurgical treatment, particularly with diazoxide, reduced the area covered by GFAP-positive processes by 33% in both SHAM and 2VO groups (Fig. 1A–C). The microglial activation in the corpus callosum displayed a tendency to be elevated due to cerebral hypoperfusion; this was manifested as a 56% increase in the non-treated 2VO group compared with the non-treated SHAM group. The solvent DMSO did not change this tendency; however, diazoxide treatment restored the microglial activation in the 2VO animals to the control level (Fig. 1D–F).

The optic tract was the most clearly affected by cerebral hypoperfusion. The astrocytic proliferation was consistently increased by about 20% in the 2VO groups as compared with their respective SHAM controls, regardless of pharmacological treatment (Fig. 2A–C). Similarly, the

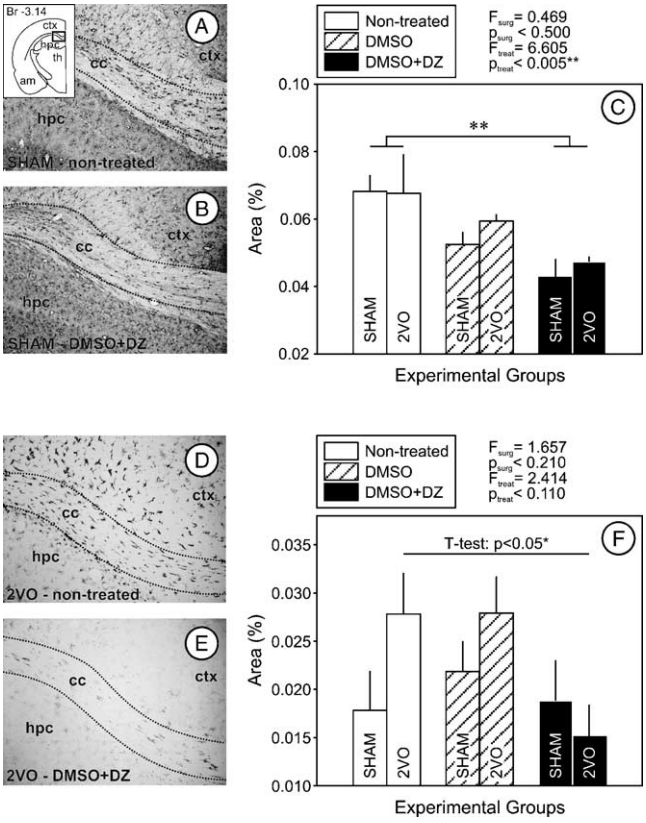

Fig. 1. Corpus callosum: photomicrographs and quantitative data on GFAP-positive astrocytic proliferation and OX-42-positive microglial activation. Panels A and B: Representative microscopic images of GFAP immunolabeling; original magnification: 10×. Panel C: Quantitative data on astrocytic proliferation; $^{**}P < 0.01$. Panels D and E: Representative microscopic images of OX-42 immunolabeling; original magnification: 10×. Panel F: Quantitative data on microglial activation. Abbreviations: 2VO: bilateral carotid artery occlusion, am: amygdala complex, cc: corpus callosum, ctx: cerebral cortex, DMSO: dimethyl sulphoxide, DZ: diazoxide, hpc: hippocampus, SHAM: sham-operated control, th: thalamus.

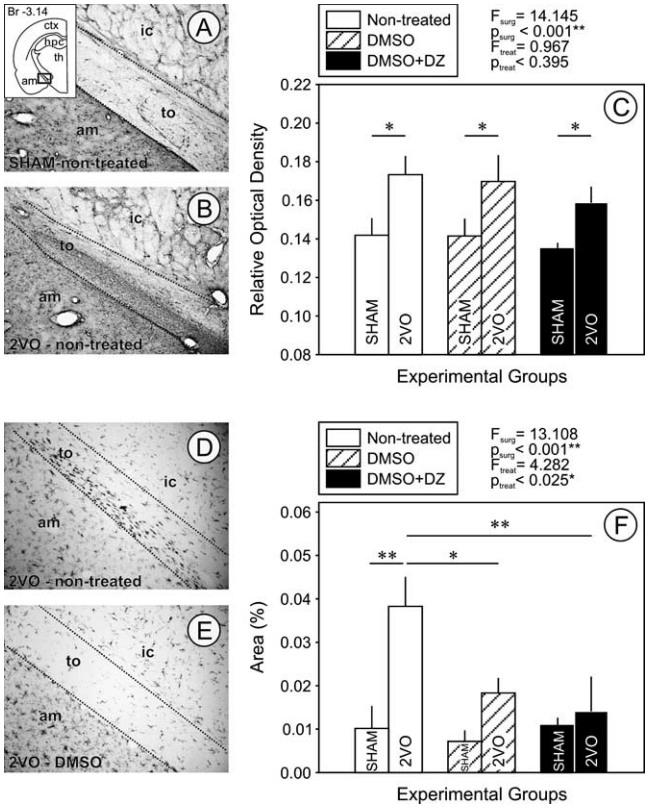

Fig. 2. Optic tract: photomicrographs and quantitative data on GFAP-positive astrocytic proliferation and OX-42-positive microglial activation. Panels A and B: Representative microscopic images of GFAP immunolabeling; original magnification: 10×. Panel C: Quantitative data on astrocytic proliferation; $^{*}P < 0.05$. Panels D and E: Representative microscopic images of OX-42 immunolabeling; original magnification: 10×. Panel F: Quantitative data on microglial activation; $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: 2VO: bilateral carotid artery occlusion, am: amygdala complex, ctx: cerebral cortex, DMSO: dimethyl sulphoxide, DZ: diazoxide, hpc: hippocampus, ic: internal capsule, SHAM: sham-operated control, th: thalamus.

microglial activation was markedly enhanced in the non-treated 2VO group as compared with the corresponding SHAM group, but both DMSO and diazoxide restored the microglial activation in the 2VO animals to the SHAM level (Fig. 2D–F).

Thus, both cerebral hypoperfusion and the pharmacological treatment elicited region-specific changes in astrocyte proliferation and microglial activation. The optic tract was predominantly vulnerable to cerebral hypoperfusion, while diazoxide dissolved in DMSO preferentially exerted an effect in the corpus callosum.

The findings reported here primarily confirm our previous observation that chronic cerebral hypoperfusion leads to astrocytic proliferation and microglial activation, specifically in the optic tract [8]. As noted earlier, the varying degree of blood supply to the different white matter areas in the rat brain may be responsible for the regional specificity, the optic tract receiving a direct branch of the internal carotid artery originating bellow the level of the circle of Willis [15], which makes flow compensation to the optic tract after common carotid artery occlusion improbable.

The pharmacological treatment applied here also appeared to exert region-specific effects. Diazoxide dissolved in DMSO reduced the astrocytic proliferation in the corpus callosum, but not in the optic tract, regardless of the degree of cerebral perfusion. Diazoxide in DMSO also decreased the ischemia-induced microglial activation specifically in the corpus callosum, but not in the optic tract. The pattern of microglial activation in the corpus callosum, with or without drug treatment, appeared to be very similar to that previously seen in the adjacent hippocampus [9].

There can be many reasons why diazoxide detectably attenuated the glial reaction only in the corpus callosum of the three white matter areas investigated. In the case of microglial activation, DMSO could possibly obscure the effect of diazoxide in the optic tract, but not in the corpus callosum, and it could be DMSO rather than diazoxide that differentially affected the various white matter areas. Conversely, the

different reactions of the astrocytes in the examined white matter regions to diazoxide treatment may stem from the region-specific sensitivity/composition of the mitochondrial and surface cation channels of the astrocytes, as detailed below.

The mechanism behind the action of diazoxide on the astrocytes is a matter of debate. When cultured astrocytes were incubated with diazoxide, loss of mitochondrial membrane potential, an elevated free radical production, and protein kinase C activation were observed. These results suggested that mitochondrial ATP-sensitive potassium channels served as the target for diazoxide in the astrocytes [13]. On the other hand, another recent investigation has revealed the possibility that ATP-sensitive non-selective cation channels on the surface of reactive astrocytes could be regulated by sulfonylurea receptor-1, which is the site of action of diazoxide [3]. These data suggest that diazoxide can target both mitochondrial and surface cation channels on the astrocytes, thereby modulating the astrocytic function, which could explain our present data.

Very little is known about the effect of diazoxide on the microglia. We published the first report that diazoxide dissolved in DMSO may suppress ischemia-related microglial activation in the rat hippocampus [9]. Our present data reveal a similar tendency in the corpus callosum. Although the resting/active state of the microglia is defined by the cell surface potassium, proton and chloride channels [6,10,21], there is no experimental evidence that demonstrates whether sulfonylurea receptors (the target of diazoxide) play a role in the regulation of these ion channels. It is not clear either how the binding of diazoxide to the mitochondrial ATP-sensitive potassium channels can alter the microglial activity, since no direct causal link has been established between mitochondrial function and microglial activity. The intracellular pathways of the effects of diazoxide on the microglial functions therefore remain to be clarified.

In summary, the present results indicate that pharmacological intervention can attenuate ischemia-related white matter injury. The diazoxide and DMSO treatment applied here was specifically effective in suppressing glial reactivity. Such attenuation of astrocytic proliferation and microglial activation tends to weaken the ischemia-induced neuroinflammatory responses, and prevent the progression of white matter lesions.

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